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Degradation of Cyclin A Does Not Require Its Phosphorylation by CDC2 and Cyclin-dependent Kinase 2*

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Many cyclins are degraded by the ubiquitination/proteasome pathways involving the anaphase-promoting complex and SCF complexes. These degradations are frequently dependent on phosphorylation by cyclin-dependent kinases (CDKs), providing a self-limiting mechanism for CDK activity. Here we present evidence from *in vitro* and *in vivo* assay systems that the degradation of human cyclin A can be inhibited by kinase-inactive mutants of CDK2 and CDC2. One obvious interpretation of these results is that like other cyclins, CDK-dependent phosphorylation of the cyclin A may be involved in cyclin A degradation. Our data indicated that CDK2 can phosphorylate cyclin A on Ser-154. Site-directed mutagenesis of Ser-154 abolished the phosphorylation by recombinant CDK2 *in vitro* and the majority of cyclin A phosphorylation in the cell. Activation of CDK2 and binding to SKP2 or $p27^{KIP1}$ were not affected by the phosphorylation of Ser-154. Surprising, in marked contrast to cyclin E, where phosphorylation of Thr-380 by CDK2 is required for proteolysis, degradation of cyclin A was not affected by Ser-154 phosphorylation. It is likely that the stabilization of cyclin A by the kinase-inactive CDKs was mainly due to a cell cycle effect. These data suggest an important difference between the regulation of cyclin A and cyclin E.

Cyclins and cyclin-dependent kinases (CDKs)[1] are key regulators of the eukaryotic cell cycle. Cyclin B is associated with CDC2 and the cyclin B-CDC2 complexes regulate entry into M phase (1). Cyclin A-CDK2 and cyclin E-CDK2 complexes are important for progression through S phase and the G_1/S transition, respectively (2, 3). D-type cyclins are associated with CDK4 and CDK6, and the complexes are required for G_1 progression (2).

The kinase activity of CDK is tightly regulated by an intricate system of phosphorylation and protein-protein interactions (4, 5). By definition, the activation of CDKs is dependent on the association with a cyclin subunit. The post-translational regulation of cyclins occurs mainly through degradation (6). The mitotic cyclins are degraded near the end of anaphase by the ubiquitin-proteasome system, consisting of a nonspecific ubiquitin-activating enzyme, a ubiquitin-carrier protein, a cyclin-specific ubiquitin ligase known as the cyclosome or anaphase-promoting complex (APC), and a constitutively active proteasome complex. The N-terminal destruction box sequences of the mitotic cyclins are required for their degradation (7), possibly because they are recognized by the cyclin-specific ubiquitin ligase enzyme. Recent biochemical and genetic studies have demonstrated that vertebrate APC contains at least eight subunits (APC1–APC8) (8).

The activity of APC is highly regulated, turning on in anaphase and persist until late G_1 (9, 10). Association between APC and the WD40 repeat-containing protein Hct1p (also known as Cdh1p) is required for the degradation of mitotic cyclins in yeast (11). From S phase onward, phosphorylation of Hct1p by CDKs blocked the Hct1p-APC interaction (12), allowing the mitotic cyclins to accumulate until anaphase. After anaphase, cyclin destruction is initiated by the dephosphorylation of Hct1p by the phosphatase Cdc14p (13, 14). Cdc14p is sequestered in the nucleolus by anchoring to Cfi1p for most of the cell cycle and is only released from the nucleolus to act on its targets during anaphase (15).

Polo-like kinase (Cdc5p in *Saccharomyces cerevisiae*) is activated by cyclin B-CDC2, and activated Polo-like kinase phosphorylates components of APC and promotes the ubiquitination of cyclin B (16–18). Protein kinase A, on the other hand, phosphorylates two subunits of APC but suppresses APC activity (18). The high activity of Polo-like kinase and low activity of protein kinase A at metaphase may contribute to the activation of APC. Cdc5p itself is degraded by Hct1p-APC-dependent mechanism in G_1, which could provide a feedback mechanism by which the APC destroys its activator at the onset of the next cell cycle.

Unlike the mitotic cyclins, the G_1 cyclins do not have a destruction box but do contain PEST sequences at the C-terminal portion of the protein that are partly responsible for the relatively short half-life of these cyclins (19). In yeast, CDK-dependent phosphorylation of the Cln is involved in the ubiquitination-dependent turnover of these cyclins (20, 21). Similarly, phosphorylation of human cyclin E (on Thr-380) and cyclin D1 (on Thr-286) is important for their ubiquitin-dependent degradation (22–24). Interestingly, the CDK2 partner of cyclin E can phosphorylate cyclin E at Thr-380, but the CDK4 partner of cyclin D1 is apparently not responsible for cyclin D1 Thr-286 phosphorylation. Instead, Thr-286 can be phosphorylated by GSK-3β, linking cyclin D1 turnover to mitogen signal transduction pathways (25).

The ubiquitination of Cln cyclins involves their phosphorylation-dependent association with SCF complexes. The current model is that cullins (Cdc53p in yeast), together with SKP1, RBX1/ROC1 (or Hrt1p), and an F box-containing protein, assemble into a cyclin-specific ubiquitin ligase complex named

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; APC, anaphase-promoting complex; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; CAK, CDK-activating kinase.

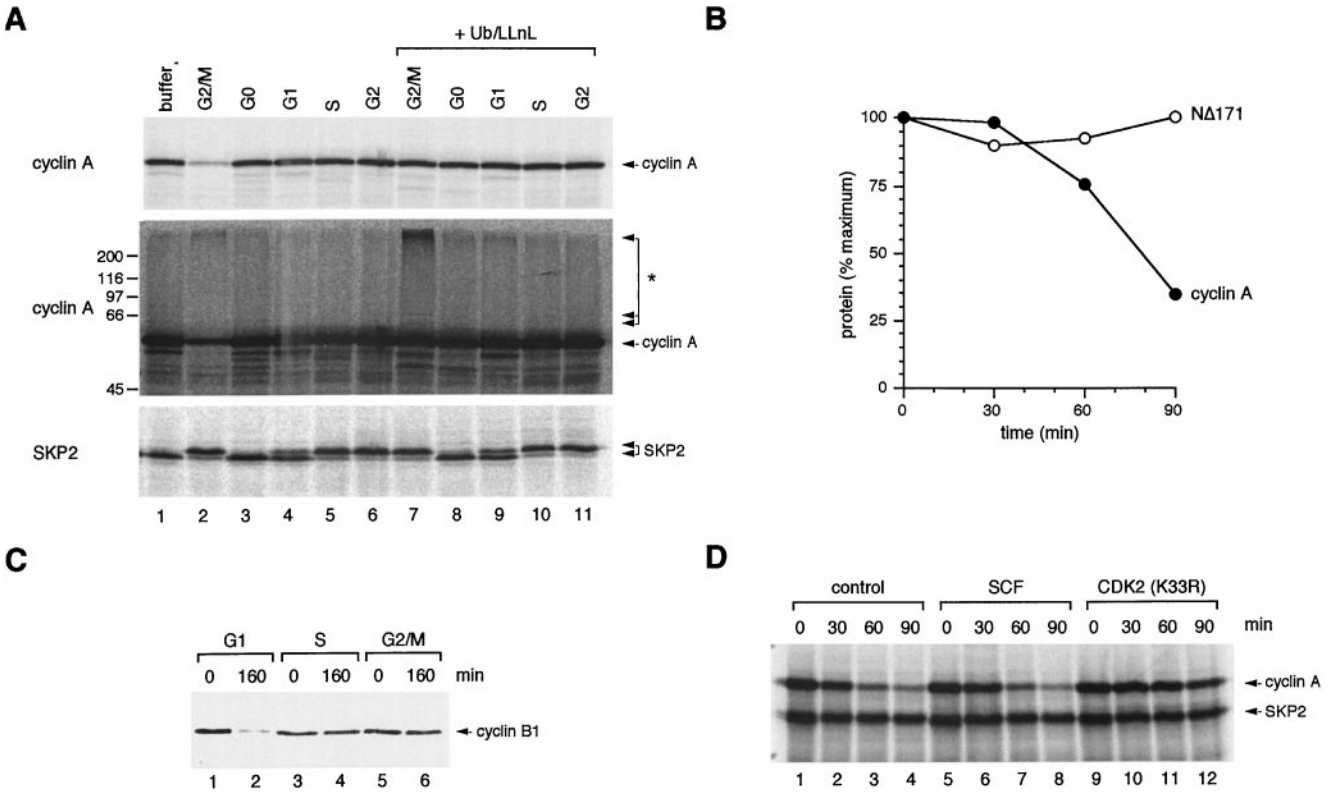

FIG. 1. **Destruction of cyclin A *in vitro* was inhibited by kinase-inactive CDK2.** *A,* destruction of cyclin A by nocodazole-blocked extracts. Human cyclin A translated in reticulocyte lysate in the presence of [^{35}S]methionine was mixed with buffer (*lane 1*) or extracts derived from cells in G_2/M blocked by nocodazole (*lanes 2* and *7*), G_0 (*lanes 3* and *8*), G_1 (*lanes 4* and *9*), S (*lanes 5* and *10*), and G_2 (*lanes 6* and *11*). G_0 cell extracts were from Swiss 3T3 cells, and other cell extracts were from HeLa cells. LLnL and ubiquitin were added to the reactions in *lanes 7–11.* The reactions were incubated at 30 °C for 90 min, followed by SDS-PAGE and detection with a PhosphorImager (*upper panel*). Higher exposure of the same gel is shown in the *middle panel.* Molecular size markers are shown on the *left,* and the larger forms of the cyclin A are indicated by the *asterisk.* The same reactions were performed with human SKP2 instead of cyclin A in the *bottom panel. B,* cyclin A(NΔ171) is stable. Reticulocyte lysate-produced cyclin A or cyclin A(NΔ171) was mixed with cell-free extracts derived from nocodazole-blocked HeLa cells. The reactions were incubated at 30 °C and harvested at various time points. The samples were applied onto SDS-PAGE, and the amount of [^{35}S]labeled cyclin A and cyclin A(NΔ171) was quantitated with a PhosphorImager. *C,* destruction of cyclin B1 *in vitro*. Reticulocyte lysate-produced human cyclin B1 was mixed with extracts derived from cells in G_1 (*lanes 1* and *2*), S (*lanes 3* and *4*), or G_2/M (nocodazole-blocked) (*lanes 5* and *6*). The reactions were incubated at 30 °C and harvested at 0 and 160 min as indicated. The samples were applied onto SDS-PAGE, and the amount of ^{35}S-labeled cyclin B1 was detected with a PhosphorImager. *D,* reticulocyte lysate-produced cyclin A and SKP2 were mixed with nocodazole-blocked HeLa extracts, in the presence of extracts derived from cells transfected with control vector (*lanes 1–4*), SCF^{SKP2} (SKP1, SKP2, and CUL1) (*lanes 5–8*), or CDK2(K33R) (*lanes 9–12*). The reactions were incubated at 30 °C and harvested at the indicated time points for SDS-PAGE and PhosphorImagery. The positions of cyclin A and SKP2 are indicated.

SCF. CDC34 (ubiquitin-carrier protein enzyme) and SCF complexes containing the same SKP1, cullin, and RBX1/ROC1 but different F box-containing proteins may target different proteins for ubiquitination. For the Cln cyclins, SCF^{GRR1} binds to phosphorylated Cln1p and Cln2p and targets their destruction (26–31).

Cyclin A is one of the first cyclins to be identified and is believed to function between that of cyclin E and cyclin B. Cyclin A can associate with both CDK2 (also a cyclin E partner) and CDC2 (also a cyclin B partner) in somatic cells. Two forms of A-type cyclins, cyclin A1 and cyclin A2, which represent the embryonic and somatic A-type cyclins, respectively, are present in higher eukaryotes. Disruption of cyclin A1 in mice leads to a block of the first meiotic division in male mice (32). On the other hand, disruption of cyclin A2 causes early embryonic lethality (33). Cyclin A-CDK2 associates with the SCF^{SKP2} complex (34–36). SKP2 is overexpressed in many cancer cells (37, 38), but the precise function of cyclin A-CDK2-SCF^{SKP2} complex is unclear. One possibility is that cyclin A-CDK2 that binds SCF^{SKP2} phosphorylates substrates that are subsequently targeted for degradation by the SCF^{SKP2} complex, since there is a propensity for SCF complexes to interact only with the phosphorylated forms of their targets. One potential target of SCF^{SKP2} is $p27^{KIP1}$; SCF^{SKP2} interacts with the Thr-187-phosphorylated form of $p27^{KIP1}$ and targets $p27^{KIP1}$ for ubiquitination (39). Degradation of the transcription factor E2F-1 has also been linked to its interaction with SCF^{SKP2} (40), but phosphorylation of E2F-1 is apparently not required in this case.

Given that the degradation of mammalian cyclin D, cyclin E, and the yeast Cln cyclins is linked to phosphorylation by CDKs and other kinases, it is interesting to see whether the degradation of the mammalian cyclin A is also linked to its phosphorylation by CDKs. By using *in vitro* and *in vivo* degradation assays, we showed that the degradation of cyclin A can be inhibited by a kinase-inactive mutant of CDK2, a kinase-inactive mutant of CDC2, and the CDK inhibitor $p21^{CIP1/WAF1}$. Since one obvious explanation of these results is that cyclin A degradation may involve CDK-dependent phosphorylation of cyclin A, we next identified the CDK phosphorylation site in cyclin A as Ser-154. Surprising, we found that mutation of the cyclin A phosphorylation site to alanine did not affect the degradation of cyclin A. Degradation of cyclin E was also inhibited by kinase-inactive CDK2. But in contrast to cyclin A, mutation of the CDK phosphorylation site in cyclin E (Thr-380) to alanine inhibited the degradation of cyclin E.

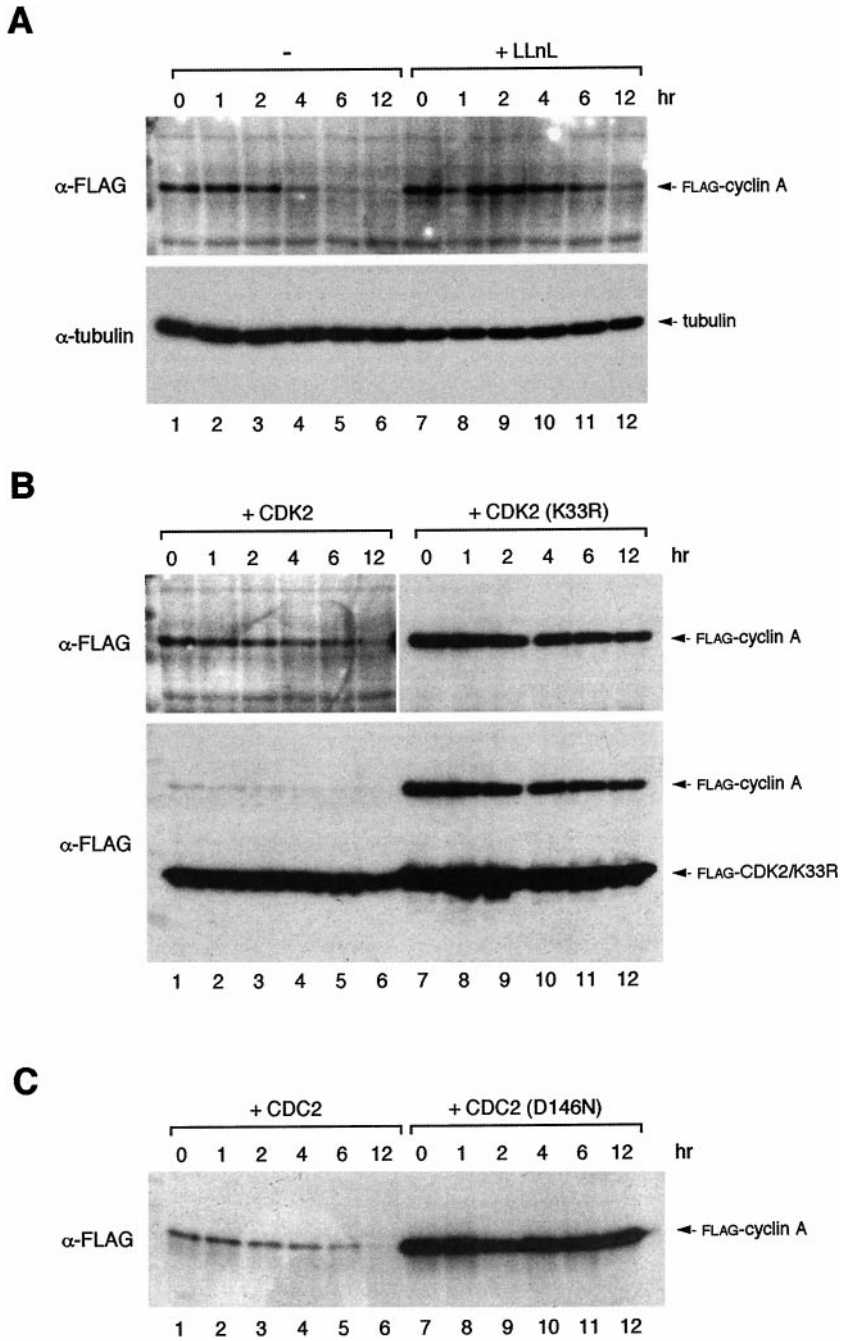

FIG. 2. **Cyclin A is stabilized by kinase-inactive CDK2 and CDC2.** *A,* HtTA1 cells were transfected with plasmids expressing FLAG-cyclin A. Buffer (*lanes 1–6*) or LLnL (*lanes 7–12*) was added to the medium, and the expression of FLAG-cyclin A was turned off by the addition of deoxycycline as described under "Materials and Methods." Samples were harvested at the indicated time and subjected to immunoblotting with antibodies against the FLAG (*upper panel*) and tubulin (*lower panel*). *B,* plasmids expressing FLAG-cyclin A were transfected with plasmids expressing FLAG-CDK2 (*lanes 1–6*) or FLAG-CDK2(K33R) (*lanes 7–12*) into HtTA1 cells. Deoxycycline was added at $t = 0$, and samples were harvested at the indicated time. The samples were subjected to immunoblotting with antibodies against FLAG. The same exposure for all the samples is shown in the *bottom panel*. In the *upper panel*, the exposure of the blot with FLAG-CDK2 was higher than that with FLAG-CDK2(K33R). *C,* plasmids expressing FLAG-cyclin A were transfected with plasmids expressing FLAG-CDC2 (*lanes 1–6*) or FLAG-CDC2(D146N) (*lanes 7–12*) into HtTA1 cells. Deoxycycline was added at $t = 0$, and samples were harvested at the indicated time. The samples were subjected to immunoblotting with anti-FLAG antibodies.

MATERIALS AND METHODS

DNA Constructs—All cyclin A used in this study were from human somatic cyclin A2. Cyclin A in pGEM4 (a gift from Dr. Tong Hunter, Salk Institute) was amplified by PCR with the primers 5′-GCAGCCATGGTGGGCAACTCT-3′ (cyclin A forward) and 5′-TGAATTCTTACAGATTTAGTGTCTCTGG-3′ (cyclin A reverse), cut with *Nco*I-*Eco*RI, and put into pUHD-P1 (37) (to make FLAG-cyclin A in pUHD-P1) or pET21d (to make cyclin A in pET21d). The *Nco*I-*Xho*I fragment of cyclin A in pET21d was put into pGEX-KG to create GST-cyclin A in pGEX-KG. Site-directed mutagenesis of cyclin A was constructed by a PCR method as described elsewhere (41), using cyclin A forward and cyclin A reverse primers and the oligonucleotide 5′-AGTTTTGAGGCACCACATA-3′ and its antisense to introduce the mutation; the PCR product was cut with *Nco*I-*Eco*RI and ligated into pUHD-P1 or pET21d. Cyclin A in pET21d was cut with *Nco*I-*Xho*I and put into pGEX-KG to create GST-cyclin A(S154A) in pGEX-KG. Cyclin A(NΔ171) in pET21d was a gift from Dr. Tim Hunt (ICRF, UK). H10-Protein A-cyclin A in pET16b was as described previously (42).

Human cyclin B1 in pGEM4 (a gift from Dr. Tong Hunter, The Salk Institute) was cut with *Nco*I and *Eco*RI, and put into pET21d to give cyclin B1-H6 in pET21d. Cyclin E in pBluescriptII(KS) was amplified by PCR with the oligonucleotides 5′-CCCGATCGATTAATACGACTCACTATAGGG-3′ and 5-′CGATATCGATATTAACCCTCACTAAAGGGA-3′, introducing the *Cla*I sites; the *Cla*I-cut fragment was put into the vector pLINX (a gift from Dr. Wei Jiang, The Salk Institute). Site-directed mutagenesis of cyclin E was as cyclin A above, using the oligonucleotide 5′-GGGCTCCTCGCCCCGCCACA-3′ and its antisense to introduce the mutation; the PCR product was put into *Cla*I-cut pLINX to give cyclin E(T380A) in pLINX. Human cyclin F in pET11d was a gift from Dr. Tim Hunt (ICRF, UK). Cyclin F in pET11d was cleaved with *Nco*I-*Eco*RI and ligated into pUHD-P1 to produce FLAG-cyclin F in pUHD-P1.

FLAG-CDK2 in pUHD-P1 and FLAG-CDK2(K33R) in pUHD-P1 were created by putting the *Nco*I-*Eco*RI fragments of GST-CDK2 in pGEX-2T and GST-CDK2 (K33R) in pGEX-KG (42), respectively, into pUHD-P1. CDC2 and CDC2(D146N) in pCMV were gifts from Dr. Wei Jiang (The Salk Institute). GST-CDK2 and GST-Cak1 on the same plasmid for co-expression in bacteria were gifts from Dr. Jane Endicott (University of Oxford, UK).

FLAG-SKP1 in pUHD-P1 and FLAG-SKP2 in pUHD-P1 were as described previously (37). GST-SKP1 in pGEX-KG and GST-SKP2 in

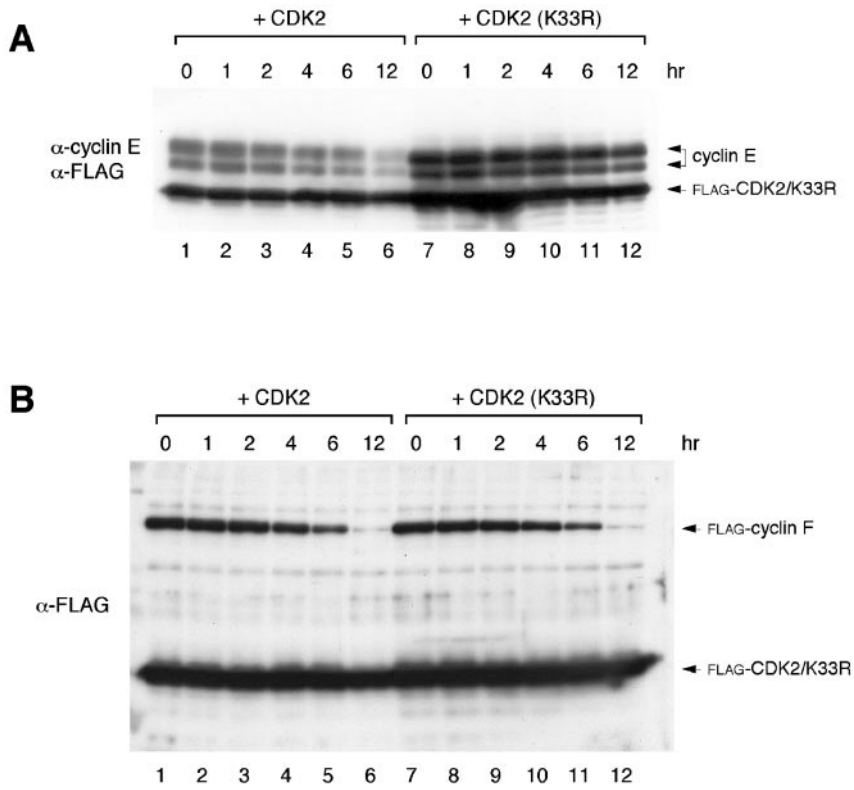

FIG. 3. **Destruction of cyclin E, but not cyclin F, was inhibited by kinase-inactive CDK2.** *A,* cyclin E-expressing plasmids were transfected with FLAG-CDK2 (*lanes 1–6*) or FLAG-CDK2(K33R) (*lanes 7–12*) plasmids into HtTA1 cells. Deoxycycline was added at $t = 0$, and samples were harvested at the indicated time. The samples were subjected to immunoblotting for cyclin E and FLAG. Note that cyclin E here was not tagged, hence there was some residual endogenous cyclin E signal even at 12 h. *B,* plasmids expressing FLAG-cyclin F were transfected with plasmids expressing FLAG-CDK2 (*lanes 1–6*) or FLAG-CDK2(K33R) (*lanes 7–12*) into HtTA1 cells. Deoxycycline was added at $t = 0$, and samples were harvested at the indicated time. The samples were subjected to immunoblotting with anti-FLAG antibodies.

pGEX-KG (37) were amplified by PCR with the primers 5′-GACCCAATGTGCCTGGATGCG-3′ and 5′-TTTCCATGGTCATCACCGAAACGCGCGAG-3′, cleaved with *Nco*I, and ligated into pUHD-P2 (a gift from Dr. Kun Ping Lu, Harvard Medical School) to produce HA-SKP1 in pUHD-P2 and HA-SKP2 in pUHD-P2, respectively. SKP2 in pET21d was as described previously (37). FLAG-CUL1 in pUHD-P1 was constructed by putting the CUL1 cDNA PCR fragment (with oligonucleotides 5′-CGGATCCATGTCGTCAACCCGGAGCCAGAACC-3′ and 5′-CGGATCCTTAAGCCAAGTAACTGTAGGTGTC-3′) into the *Bam*HI site of pUHD-P1.

FLAG-p21 in pcDNA3.1(−) was constructed by putting the *Nhe*I-*Xba*I fragment of FLAG-p21 in pUHD-P1 (43) into pcDNA3.1(−) (Invitrogen). Human p27 in pLINX was created by amplification of the p27-H6 in pET21a (44) by PCR using the oligonucleotides 5′-CCCGATCGATTAATACGACTCACTATAGGG-3′ and 5′-AGGGATCGATCTAGTTATTGCTCAGCGGTGG-3′, followed by cutting with *Cla*I and ligation into pLINX. Human B-cell antigen CD20 in pCMX was as described previously (37). Histidine-tagged ubiquitin for bacterial expression was a gift from Tim Hunt (Imperial Cancer Research Fund, UK).

Cell Culture—HtTA1 cells (gifts from Dr. Hermann Bujard) are HeLa cells (human cervical carcinoma) stably transfected with pUHD15–1 expressing the tTA tetracycline repressor chimera (45) and can express genes cloned into the pUHD-P1, pUHD-P2, or pLINX vectors in the absence of deoxycycline (1 μg/ml to turn off expression). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum in a humidified incubator at 37 °C in 10% CO_2. Serum-starved Swiss 3T3 cells were obtained as described previously (44). LLnL (also called MG101) was used at 50 μM for 16 h. Cells were blocked at G_2/M by incubation in medium containing 0.1 μg/ml nocodazole for 16 h. G_1 cells were obtained 4 h after released from the nocodazole block. Cells were blocked in S phase by incubation in medium containing 2 mM hydroxyurea for 24 h.

Semiconfluent HtTA1 cells (10-cm diameter plates) were transiently transfected with constructs (10 μg) by the calcium phosphate method (46). The total amount of DNA for each transfection was adjusted to the same level using vectors containing the same promoter. Cells were grown for a further 24 h after transfection before being harvested for cell extracts. For preparation of cell extracts for destruction assays, cells were lysed in a hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol) and sonicated on ice for 5 min. The lysate were frozen at −80 °C, then thawed on ice, and centrifuged in a microcentrifuge for 10 min to pellet the insoluble materials. For other purposes, cell extracts were prepared with a Nonidet P-40 lysis method as described elsewhere (44). The protein concentration of cell lysates was measured with a bicinchoninic acid protein assay system (Pierce), using bovine serum albumin as standards. In promoter turn-off experiments, two 10-cm dishes of cells were transfected with the indicated plasmids as described above. At 16 h after transfection, the cells were washed with PBS, and split into six identical 60-mm plates. After growing for another 24 h, deoxycycline (1 μg/ml) was added to the medium, and the cells were harvested at the indicated time.

Flow Cytometry—Flow cytometry analysis was essentially as described previously (37, 47). Semiconfluent cells were transiently transfected with a vector expressing CD20 surface marker (2 μg). After transfection (16 h), the cells were washed with phosphate-buffered saline (PBS) and grown in fresh medium for another 24 h. Cells were then trypsinized, washed with PBS, and incubated with fluorescein isothiocyanate-conjugated anti-CD20 monoclonal antibody (Dako) according to the manufacturer's instructions. Cells were then fixed in ice-cold 70% ethanol and stained with a solution containing propidium iodide (40 μg/ml) and RNase A (40 μg/ml) at 37 °C for 30 min. Cell cycle distribution of the CD20-positive (transfected) cells and the CD20-negative (non-transfected) cells was analyzed with a FACSort machine (Becton Dickinson).

Expression of Recombinant Proteins—Coupled transcription-translation reactions in the presence of [^{35}S]methionine in rabbit reticulocyte lysate were performed according to the manufacturer's instructions (Promega, Madison, WI). Expression and purification of GST- and histidine-tagged proteins were as described previously (42). Thrombin cleavage of fusion proteins was as described (48).

Destruction Assays—Human cyclin A translated in reticulocyte lysate in the presence of [^{35}S]methionine (1 μl) was mixed with 9 μl of buffer or nocodazole-blocked cell extracts (or other cell extracts as stated). Alternatively, when two different extracts were used, 1 μl of reticulocyte lysate was mixed with 4.5 μl of nocodazole-blocked extract and 4.5 μl of another cell extract. In some experiments, LLnL (50 μM) and ubiquitin-H6 (0.1 mg/ml) were added to the reactions. The reactions were supplemented with an energy mix (final concentration of 25 mM phosphocreatine, 10 μg/ml creatine kinase, and 1 mM ATP). Unless stated otherwise, the reactions were incubated at 30 °C for 90 min and stopped by addition of SDS-sample buffer.

Kinase Assays, Phospholabeling, and Phosphoamino Acid Analysis—Histone H1 kinase assays were performed as described (48). Two-dimensional phosphoamino acid analysis after partial acid hydrolysis was performed on $^{32}PO_4$-labeled polypeptides after transfer to Immobilon (Millipore) as described (49). Phosphoamino acids were separated on cellulose TLC plates (Schleicher & Schuell) by electrophoresis at

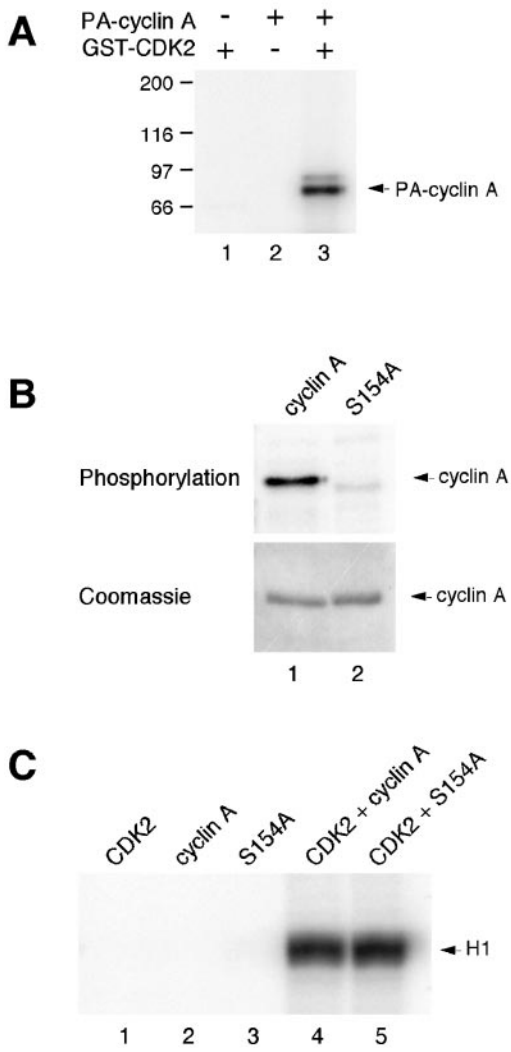

FIG. 4. **Phosphorylation of recombinant cyclin A by CDK2 *in vitro*.** *A,* bacterially expressed protein A-cyclin A (*lanes 1* and *3*) and CAK-activated GST-CDK2 (*lanes 2* and *3*) were incubated in the presence of [γ-^{32}P]ATP. The samples were applied onto SDS-PAGE, and phosphorylation was detected by PhosphorImagery. The positions of the molecular size standards are shown on the *left. B,* thrombin-cleaved GST-cyclin A (*lane 1*) and GST-cyclin A(S154A) (1 μg) were incubated with CAK-activated GST-CDK2 (0.1 μg) in the presence of [γ-^{32}P]ATP. Phosphorylation was detected by SDS-PAGE and PhosphorImagery (*upper panel*). The presence of cyclin A and S154A mutant was detected by Coomassie Blue staining (*lower panel*). *C,* the histone H1 kinase activities of CAK-activated GST-CDK2 (*lane 1*), cyclin A (*lane 2*), cyclin A(S154A) (*lane 3*), GST-CDK2 and cyclin A (*lane 4*), and GST-CDK2 and cyclin A(S154A) (*lane 5*) were assayed. Histone H1 phosphorylation was detected by SDS-PAGE followed by PhosphorImagery. *PA,* protein A.

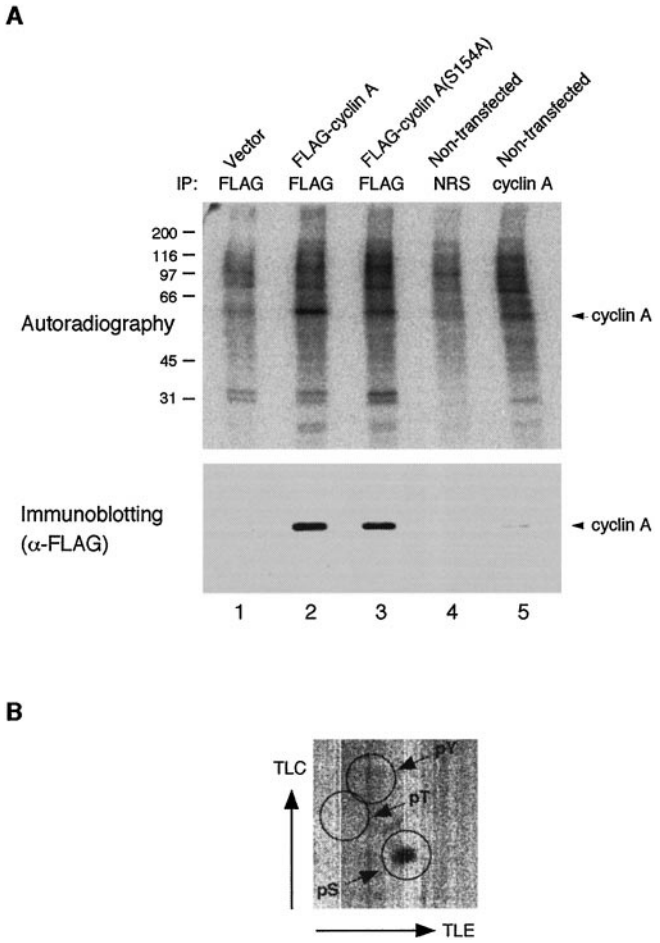

FIG. 5. **Phosphorylation of cyclin A in HeLa cells.** *A,* HtTA1 cells were transfected with control plasmids (*lane 1*), FLAG-cyclin A (*lane 2*), or FLAG-cyclin A(S154A) (*lane 3*). Transfected cells or normal growing cells (*lanes 4* and *5*) were labeled with ^{32}P for 4 h. Cell extracts were prepared and subjected to immunoprecipitation (*IP*) with anti-FLAG polyclonal antibodies (*lanes 1–3*), normal rabbit serum (*lane 4*), or anti-cyclin A polyclonal antibodies (*lane 5*). The immunoprecipitates were applied onto a 17.5% SDS-PAGE, and phosphorylations were detected with a PhosphorImager (*upper panel*). The positions of cyclin A and the molecular size standards (in kDa) are indicated. The same immunoprecipitates were subjected to immunoblotting with anti-FLAG monoclonal antibody M2 (*lower panel*). *B,* phosphoamino acid analysis of cyclin A. The phosphorylated FLAG-cyclin A band from *lane 1* above was cut out and subjected to phosphoamino acid analysis using thin layer electrophoresis (*TLE*) in the first dimension and thin layer chromatography (*TLC*) in the second dimension, followed by analysis by PhosphorImagery. The positions of phosphoamino acids standard are indicated.

1600 V for 60 min with pH 1.9 buffer, followed by thin layer chromatography using the phospho-chromatography buffer (50). To label cells with ^{32}P, cells were grown to about 80% confluent, and the medium was changed to phosphate-free Dulbecco's modified Eagle's medium supplemented with 10% v/v PBS-dialyzed calf serum. After incubation in a humidified incubator at 37 °C in 10% CO_2, the medium was changed to phosphate-free Dulbecco's modified Eagle's medium supplemented with 10% v/v PBS-dialyzed calf serum and 1 mCi/ml ^{32}P. The cells were labeled for 4 h before harvested for cell extracts preparation. To phosphorylate cyclin A by CDK2 *in vitro*, bacterially expressed protein A-cyclin A or thrombin-cleaved GST-cyclin A (10 μg/ml) were mixed with CAK-activated GST-CDK2 (10 μg/ml) in the presence of 15 mM $Mg(OAc)_2$, 30 μM ATP, and 1.5 μCi of [γ-^{32}P]ATP. The reaction was carried out at 25 °C for 30 min.

Antibodies and Immunological Methods—Monoclonal antibody M2 against FLAG tag was obtained from Eastman Koda Co. Rabbit anti-FLAG polyclonal antibodies were gifts from Dr. Katsumi Yamashita (Kanazawa University, Japan) or from Santa Cruz Biotechnology (sc-807). Monoclonal antibody G173–524 against p27^{KIP1} was from PharMingen (San Diego, CA). Monoclonal antibody 12CA5 against hemagglutinin (HA) tag was a gift from Dr. Tony Hunter (the Salk Institute), or from Roche Molecular Biochemicals. Rat monoclonal antibodies YL1/2 against mammalian tubulin (37), anti-cyclin E monoclonal antibodies HE12 (37), and anti-PSTAIRE monoclonal antibody (51) were as described previously. Immunoblotting and immunoprecipitation were performed as described previously (44).

RESULTS

Inhibition of Cyclin A Destruction by Inactive CDK2—Cyclin A (but not cyclin B) is destroyed when cells are arrested at G_2/M with drugs like nocodazole. We first investigated the degradation of cyclin A by translating cyclin A in reticulocyte lysate in the presence of [^{35}S]methionine, and we mixed the cyclin A with extracts of HeLa cells that were synchronized in different phases of the cell cycle. Fig. 1*A* shows that cyclin A was destroyed when incubated with a nocodazole-blocked ex-

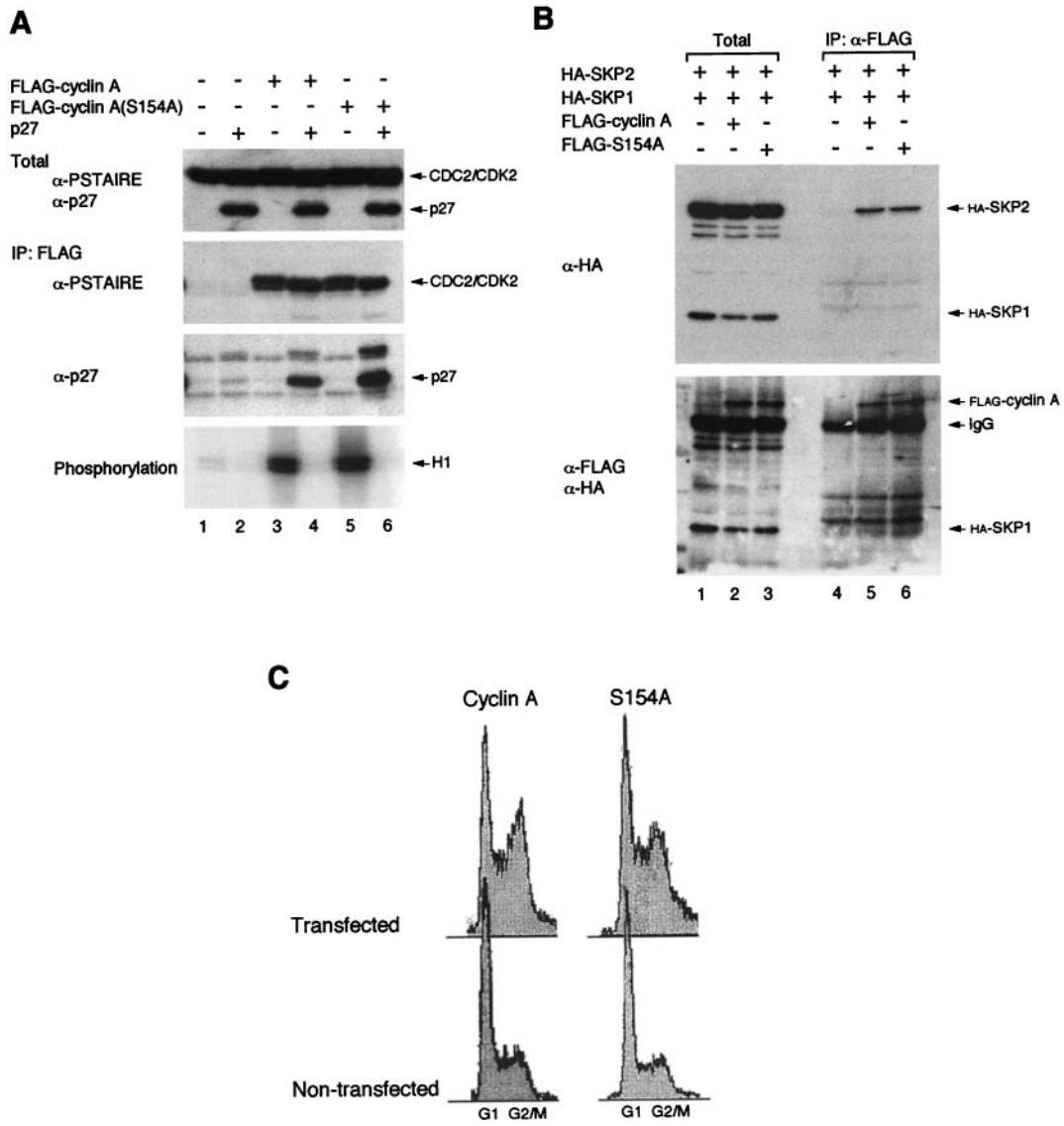

FIG. 6. **Phosphorylation of Ser-154 does not affect the activity of cyclin A.** *A,* FLAG-cyclin A (*lanes 3* and *4*), FLAG-cyclin A(S154A) (*lanes 5* and *6*), and $p27^{KIP1}$ (*lanes 2, 4,* and *6*) were transfected into HtTA1 cells. Cell extracts were prepared and 10 μg were subjected to immunoblotting with anti-PSTAIRE antibodies and anti-$p27^{KIP1}$ antibodies (*upper panel*). Extracts (200 μg) were immunoprecipitated (*IP*) with anti-FLAG polyclonal antibodies, and the immunoprecipitates were immunoblotted with antibodies against PSTAIRE or $p27^{KIP1}$ as indicated. The histone H1 kinase activities of the anti-FLAG immunoprecipitates were assayed, and phosphorylations were detected by PhosphorImagery (*lower panel*). *B,* plasmids expressing HA-SKP1 and HA-SKP2 were transfected with plasmids expressing FLAG-cyclin A (*lanes 2* and *5*) or FLAG-cyclin A(S154A) (*lanes 3* and *6*) into HtTA1 cells. Cell extracts (200 μg) were immunoprecipitated with anti-FLAG tag polyclonal antibodies. The total cell lysate (10 μg) (*lanes 1–3*) and the immunoprecipitates (*lanes 4–6*) were subjected to immunoblotting with antibodies against HA (*upper panel*), or against HA and FLAG (*lower panel*). The positions of HA- and FLAG-tagged proteins are indicated. The HA-SKP2 in the immunoprecipitations in the *lower panel* is obstructed by the IgG chains. *C,* HtTA1 cells were transfected with either cyclin A or cyclin A(S154A) and a plasmid expressing CD20. The cells were harvested 48 h after transfection. The cells were incubated with a fluorescein isothiocyanate-conjugated anti-CD20, and the DNA contents of the transfected cells (CD20-positive cells) and non-transfected cells (CD20-negative cells) were analyzed by flow cytometry. The positions of the G_1 and G_2/M DNA content are indicated.

tract (*lane 2*). In contrast, cyclin A was not destroyed when incubated with cell extracts derived from cells in G_1, S, G_2, or G_0 phase of the cell cycle. The destruction of the cyclin A by nocodazole-blocked extracts was inhibited by the addition of the calpain/proteasome inhibitor LLnL (*lane 7*). On higher exposure of the PhosphorImager (*middle panel*), higher molecular size forms of cyclin A can be seen in the presence of nocodazole-blocked extracts and LLnL. Reticulocyte lysate-translated SKP2 were used instead of cyclin A as a control (*bottom panel*), and this showed that the level of SKP2 was not altered when incubated with the nocodazole-blocked extracts. Instead, we observed mobility shifts of the SKP2 that was due to phosphorylation of Ser-76 by CDKs (37).

The destruction of cyclin A in our *in vitro* system was dependent on the presence of the N-terminal destruction box. Fig. 1*B* shows that deletion of the N terminus to residue 171 (NΔ171) inhibited the destruction of cyclin A by the nocodazole-blocked extracts. When reticulocyte lysate-translated cyclin B1 was used instead of cyclin A, we found that cyclin B1 was destroyed in G_1 phase cell extracts but not by S phase or nocodazole-blocked extracts (Fig. 1*C*). Collectively, these data indicate that we have established an *in vitro* assay for cyclin A destruction by nocodazole-blocked extracts that was specific for cyclin A but not for cyclin B1.

We next investigated whether the destruction of cyclin A by nocodazole-blocked extracts was affected by cyclin A-associated proteins. Reticulocyte lysate-produced cyclin A and SKP2 were mixed with nocodazole-blocked extracts. A third cell extracts

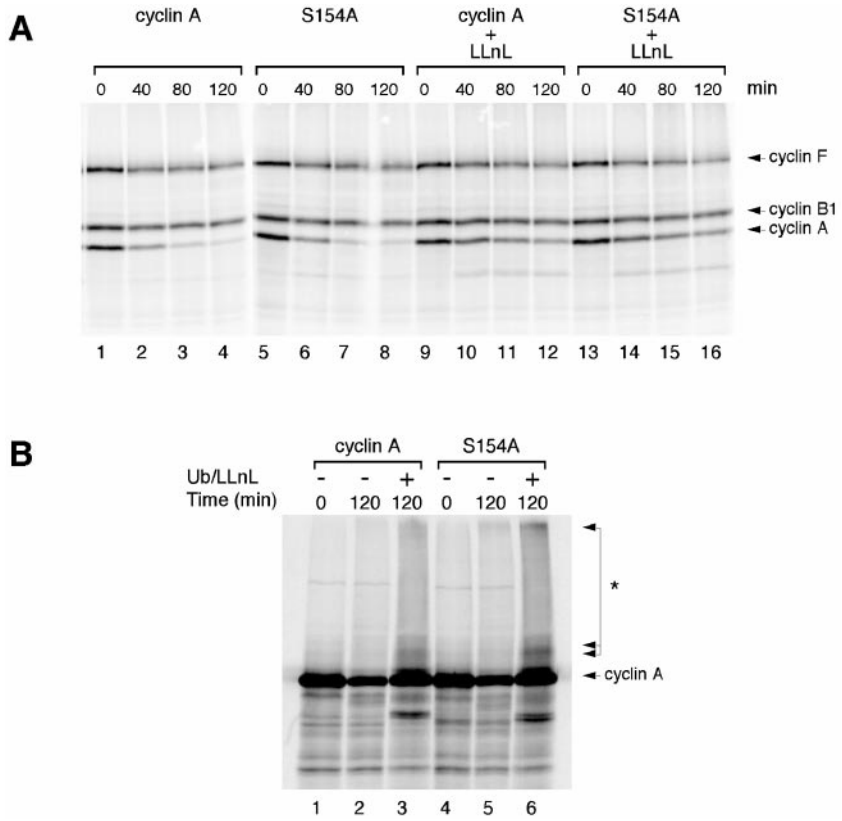

FIG. 7. **Phosphorylation of Ser-154 does not affect the half-life of cyclin A *in vitro*.** *A,* reticulocyte lysate-produced cyclin B1, cyclin F, cyclin A (*lanes 1–4*, and *9–12*), or cyclin A(S154A) (*lanes 5–8*, and *13–16*) were incubated with nocodazole-blocked HeLa extracts. LLnL was added to the reactions in *lanes 9–16.* The reactions were incubated at 30 °C, and samples were taken at the indicated time. The amount of ^{35}S-labeled proteins was detected by SDS-PAGE followed by PhosphorImagery. *B,* reticulocyte lysate-produced cyclin A (*lanes 1–3*) or cyclin A(S154A) (*lanes 4–6*) were incubated with nocodazole-blocked HeLa extracts, either in the absence (*lanes 1, 2, 4*, and *5*) or presence (*lanes 3* and *6*) of LLnL and ubiquitin. Samples were taken at $t = 0$ (*lanes 1* and *4*) or after incubation at 30 °C for 120 min (*lanes 2, 3, 5*, and *6*) and subjected to SDS-PAGE and PhosphorImagery. The higher forms of the cyclin A and S154A are indicated by the *asterisk.*

from cells transfected with either control vector or a dominant-negative kinase-dead mutant of CDK2 (K33R, Lys-33 mutated to Arg) were added to the reaction. Fig. 1*D* shows that as before cyclin A was destroyed while SKP2 control was relatively stable in the nocodazole-blocked extracts. Expression of CDK2(K33R) clearly inhibited the destruction of cyclin A (*lanes 9–12*). As a further control, expression of components of SCF^{SKP2} complexes did not significantly affect the rate of cyclin A proteolysis (*lanes 5–8*). Two likely interpretations of these results are as follows: (i) the lack of cyclin A phosphorylation by CDK2(K33R) inhibited cyclin A degradation; (ii) the expression of CDK2(K33R) blocked the cell cycle at G_1 and hence inhibited the degradation of cyclin A. The two hypotheses are related to each other, but we primarily addressed the former hypothesis in the present study. The fact that overexpression of SCF^{SKP2} also blocked the cells in G_1 phase (37) led us initially to favor the former hypothesis.

To see whether the destruction of cyclin A is affected by kinase-inactive CDK2 in more detail, we utilized a promoter turn-off assay to study the stability of cyclin A expressed in mammalian cells. HeLa cells were transfected with plasmids that expressed cyclin A under the inducible control of deoxycycline. The cells were then divided into six identical plates, and the expression of cyclin A was turned off by addition of deoxycycline to the medium. Fig. 2*A* shows that the level of cyclin A decreased over time after its promoter was turned off (*lanes 1–6*). To avoid the interference of the endogenous cyclin A in this assay (which was not turned off by deoxycycline), the transfected cyclin A was tagged with the FLAG epitope for its detection. In contrast to cyclin A, the level of tubulin remained relatively constant after deoxycycline treatment, indicating that a similar amount of extracts was loaded in each lane. As in the *in vitro* assays, FLAG-cyclin A was stabilized when LLnL was added (*lanes 7–12*). To see whether CDK2(K33R) affected the stability of cyclin A in this assay, plasmids expressing FLAG-cyclin A was co-transfected with either CDK2 or CDK2(K33R) (both were FLAG-tagged), and the stability of FLAG-cyclin A was assayed as above. Fig. 2*B* shows that the stability of cyclin A was significantly increased when expressed with CDK2(K33R) in comparison to co-expression with CDK2. Note that even at the start of the chase before the addition of deoxycycline, the level of FLAG-cyclin A was already far higher in the presence of CDK2(K33R) than in the presence of CDK2 (*lower panel*). The use of these types of promoter turn-off experiments to study the protein stability did have the limitation that the stability of the mRNA was not taken into account. However, experiments using cycloheximide to inhibit protein synthesis also produced similar results (data not shown). Taken together with the *in vitro* degradation assay, these data suggest that the stability of cyclin A can be increased in the presence of a kinase-inactive mutant of CDK2, either due to direct CDK phosphorylation or to an effect of cell cycle block.

Destruction of Cyclin A and Cyclin E, but Not Cyclin F, Is Inhibited by CDK2(K33R)—In contrast to cyclin B and cyclin E, cyclin A interacts with two different types of CDKs in the cell, CDK2 and CDC2. We next investigated whether like CDK2(K33R), a kinase-inactive version of CDC2 can also stabilize cyclin A. FLAG-cyclin A was transfected with either CDC2 or CDC2(D146N) in a similar promoter turn-off experiment as described above. Fig. 2*C* shows that the stability of cyclin A was increased in cells that were transfected with CDC2(D146N) in comparison to that transfected with CDC2. We also found that the stability of cyclin A was increased by co-expression with the CDK inhibitor p21$^{CIP1/WAF1}$ (data not shown).

As a comparison to cyclin A, we next subjected cyclin E and cyclin F to the same stability analysis. Cyclin E was significantly stabilized when expressed with CDK2(K33R) (Fig. 3*A*) or p21$^{CIP1/WAF1}$ (data not shown) in comparison to that expressed with CDK2. The half-life of cyclin E in the absence of co-transfected plasmid was similar to that co-transfected with CDK2 (data not shown). On the other hand, we found that the

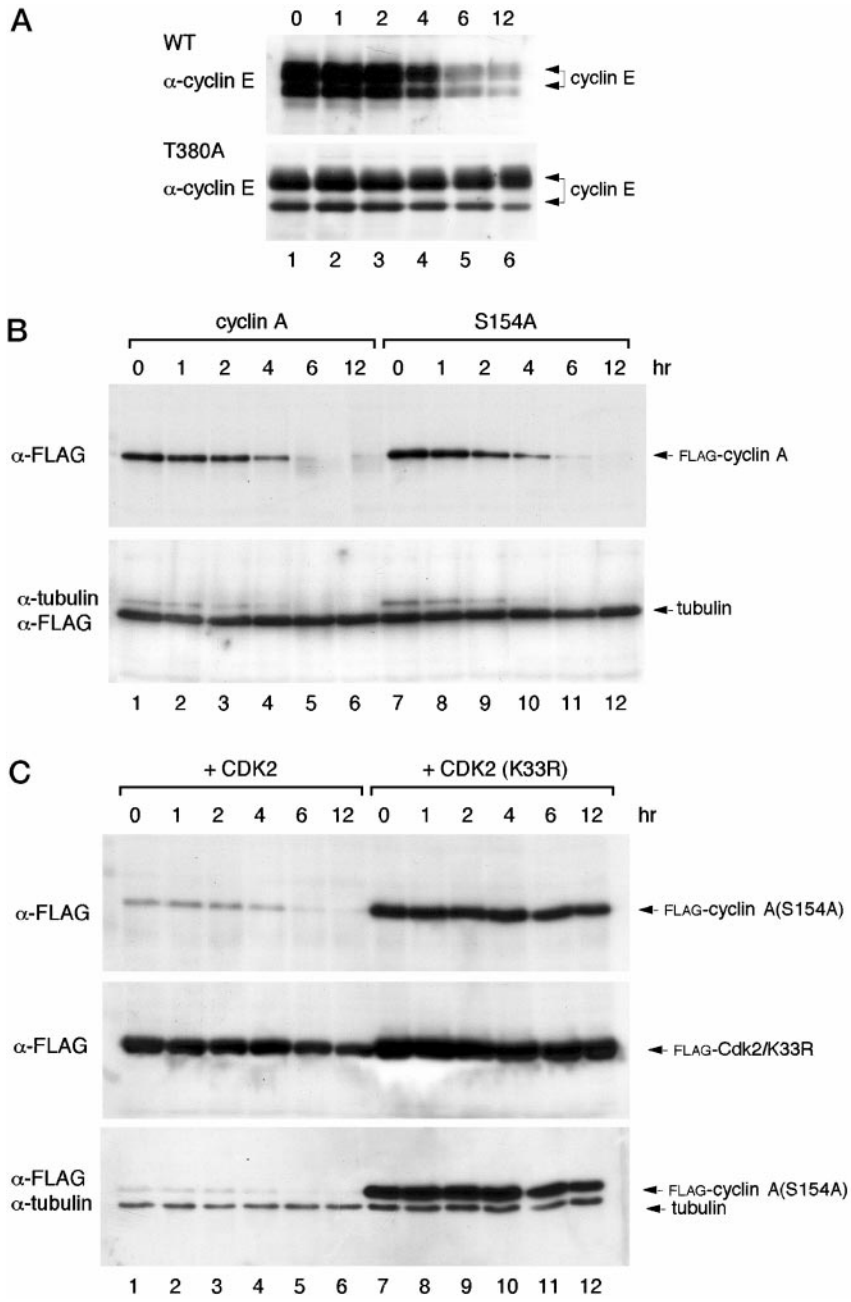

FIG. 8. **Phosphorylation of cyclin E Thr-380, but not phosphorylation of cyclin A Ser-154, is involved in the regulation of cyclin destruction.** *A,* plasmids expressing cyclin E (*upper panel*) or cyclin E(T380A) mutant were transfected into HtTA1 cells. Deoxycycline was added at $t = 0$, and samples were harvested at the indicated time. The samples were then subjected to immunoblotting with anti-cyclin E antibodies. *B,* plasmids expressing FLAG-cyclin A (*lanes 1–6*) or FLAG-cyclin A(S154A) (*lanes 7–12*) were transfected into HtTA1 cells. Deoxycycline was added at $t = 0$, and samples were harvested at the indicated time. The samples were then subjected to immunoblotting with antibodies against FLAG (*upper panel*) or FLAG and tubulin (*lower panel*). *C,* HtTA1 cells were transfected with plasmids that expressed FLAG-cyclin A(S154A) together with that expressed FLAG-CDK2 (*lanes 1–6*) or FLAG-CDK2(K33R) (*lanes 7–12*). Deoxycycline was added at $t = 0$, and samples were harvested at the indicated time. The samples were subjected to immunoblotting with anti-FLAG antibodies to detect cyclin A (*upper* and *bottom panels*) and CDK2 (*middle panel*). The bolt was probed with antibodies against FLAG and tubulin in the *bottom panel.*

stability of FLAG-cyclin F was the same whether it was expressed with CDK2 or CDK2(K33R) (Fig. 3*B*). Taken together, these experiments show that cyclin A and cyclin E, but not cyclin F, was stabilized by CDK2(K33R). This is interesting since similar to cyclin A, the endogenous cyclin F was also destroyed in nocodazole-blocked extracts,[2] but in contrast to cyclin A and cyclin E, cyclin F does not bind to CDK2.

Cyclin A Is Phosphorylated by CDK2 on Ser-154 in Vitro—We next investigated whether cyclin A can be phosphorylated by CDK2 *in vitro*. When bacterially expressed cyclin A (protein A-tagged) was incubated with CAK-activated GST-CDK2 in the presence of [γ-^{32}P]ATP, we found that the cyclin A was phosphorylated (Fig. 4*A*). No phosphorylated band at the cyclin A position was detected when GST-CDK2 or cyclin A was used individually (*lanes 1* and *2*).

On inspection of the protein sequence of human cyclin A, there is only one potential CDC2/CDK2 consensus phosphorylation site (Ser/Thr followed by a Pro) at residue Ser-154. This Ser-Pro site is conserved in the sequences of both embryonic cyclin A1 and somatic cyclin A2 in nearly all vertebrates examined. We next investigated whether Ser-154 is phosphorylated by CDK2 *in vitro*. Bacterially expressed GST fusion proteins of cyclin A and S154A mutant (with Ser-154 mutated to a non-phosphorylatable Ala) were purified, and the GST was cleaved off with thrombin. The cyclin A and S154A mutant was then incubated with CAK-activated GST-CDK2 in the presence of [γ-^{32}P]ATP. Fig. 4*B* shows that only cyclin A, but not cyclin A(S154A), was phosphorylated by CDK2. Protein staining of the gel revealed a similar amount of cyclin A and cyclin A(S154A) was present in the reactions (*lower panel*). To be sure that the lack of phosphorylation of cyclin A(S154A) was not due to a lack of CDK2 kinase activity when cyclin A(S154A) associated with CDK2, the kinase activities toward histone H1 were measured. Fig. 4*C* shows that both cyclin A-CDK2 and cyclin A(S154A)-CDK2 can phosphorylate histone H1, indicating that recombinant cyclin A(S154A)-CDK2 was as active as

[2] C. H. Yam, W. Y. Siu, A. Lau, and R. Y. C. Poon, unpublished data.

cyclin A-CDK2 complex.

Cyclin A Is Phosphorylated on Ser-154 in Vivo—We next studied whether cyclin A is also phosphorylated inside the cell. HeLa cells were labeled with ^{32}P after the cells were transfected with control plasmids, plasmids expressing FLAG-tagged cyclin A, or cyclin A(S154A) mutant. Cell extracts were prepared, and FLAG-tagged proteins were immunoprecipitated and analyzed with a PhosphorImager. Fig. 5*A* shows that FLAG-cyclin A was a phosphorylated protein in the cell (*lane 2*). Mutation of Ser-154 to Ala abolished most of the phosphorylation of cyclin A (*lane 3*), suggesting that Ser-154 was the major phosphorylation site in cyclin A. Immunoblotting of the immunoprecipitates indicated that cyclin A and cyclin A(S154A) were expressed at similar levels. In agreement with Ser-154 as the major phosphorylation site, phosphoamino acid analysis of the cyclin A bands indicated that the majority of the phosphorylation was on serine residues (Fig. 5*B*). We found that the residual phosphorylation of the S154A mutant was mainly on serine (data not shown). The endogenous cyclin A was also a phosphorylated protein in the cell (*lanes 4* and *5*), and we found that the phosphorylation was mainly on serine residues (data not shown).

Phosphorylation on Ser-154 Is Not Required for the Activity of Cyclin A—To see whether cyclin A(S154A) can bind and activate endogenous CDKs, FLAG-tagged cyclin A or cyclin A(S154A) was expressed in HeLa cells and immunoprecipitated with an anti-FLAG antibody. Fig. 6*A* shows that CDK2 and related proteins can be detected in the immunoprecipitates with an anti-PSTAIRE antibody (*lanes 3* and *5*). Furthermore, histone H1 kinase activities can be detected to associate with both FLAG-cyclin A and S154A mutant. When the CDK inhibitor $p27^{KIP1}$ was expressed with the cyclin A, complexes between $p27^{KIP1}$ and both types of cyclin A can be detected (*lanes 4* and *6*). Moreover, the kinase activity associated with both types of cyclin A was abolished by $p27^{KIP1}$, with a concomitant mobility shift of the CDKs that associated with the cyclins. These data indicate that cyclin A(S154A) can bind to CDKs and $p27^{KIP1}$.

Given that phosphorylated cyclin A could be a substrate for SCF^{SKP2}, we next investigated whether the S154A mutant can associate with SKP2. Fig. 6*B* shows that SKP2 can be co-immunoprecipitated with both cyclin A and cyclin A(S154A) (*upper panel, lanes 5* and *6*). In contrast, SKP2 was not precipitated when cyclin A was not expressed (*lane 4*). The presence of cyclin A and cyclin A(S154A) was confirmed by immunoblotting with anti-FLAG antibody (*lower panel*). This experiment shows that SKP2 can associate with cyclin A irrespective of whether it can be phosphorylated on Ser-154. We were not able to detect any HA-SKP1 binding to cyclin A-SKP2 in this experiment; this is likely due to the high level of endogenous SKP1 in the cell.

Ectopically expressed cyclin A can delay the cell cycle in G_2 or M phase. We transfected HeLa cells with cyclin A or cyclin A(S154A) and a CD20 surface maker for the subsequent selection of the transfected cells. After 48 h, the cell cycle distribution of the transfected cells (CD20-positive) and non-transfected cells was analyzed by flow cytometry. Fig. 6*C* shows that expression of cyclin A or cyclin A(S154A) resulted in a similar increase in the proportion of cells in G_2 or M phase of the cell cycle. These data suggest that at least under ectopically expressed conditions, the effects of wild type and non-phosphorylatable mutant of cyclin A on CDK activity, SKP2 binding, and on the cell cycle were the same.

In Contrast to Cyclin E, Phosphorylation of Cyclin A by CDK2 Does Not Affect the Destruction of Cyclin A—We next investigated whether the destruction of cyclin A was affected by the Ser-154 phosphorylation. We first assayed the destruction of cyclin A(S154A) using the *in vitro* system as described in Fig. 1. Reticulocyte lysate-produced cyclin A or cyclin A(S154A) was incubated with nocodazole-blocked extracts, and samples were taken at different time points for analysis. As controls, cyclin B1 and cyclin F were also mixed in the same reactions. Fig. 7*A* shows that cyclin A was destroyed, whereas both cyclin B1 and cyclin F were relatively stable (*lanes 1–4*). Surprisingly, no significant difference was observed when cyclin A(S154A) was used instead of cyclin A (*lanes 5–8*). Furthermore, LLnL inhibited the destruction of both types of cyclin A (*lanes 8–16*), and slower mobility forms were observed for both types of cyclin A in the presence of nocodazole-blocked extracts and LLnL (Fig. 7*B*). These data suggest that the ubiquitination and destruction of cyclin A were similar regardless of whether Ser-154 can be phosphorylated or not.

We next investigated the destruction of cyclin A(S154A) with the promoter turn-off assay described above. As a control, we first used cyclin E and the cyclin E(T380A) mutant (with Thr-380 mutated to an Ala) under the same deoxycycline control system. Fig. 8*A* shows that cyclin E(T380A) was more stable than cyclin E. These results show that at least our promoter turn-off assay was capable of detecting differences in the stability of cyclins due to phosphorylation. In contrast, similar half-lives were observed between cyclin A and cyclin A(S154A) (Fig. 8*B*). Moreover, we found that cyclin A(S154A) can still be stabilized when expressed with CDK2(K33R) (Fig. 8*C*). These data indicate that unlike cyclin E, where phosphorylation by CDK2 on Thr-380 is required for its degradation, phosphorylation of cyclin A on Ser-154 by CDK2 is unlikely to be involved in the ubiquitination and degradation of cyclin A.

DISCUSSION

Here we show that cyclin A can be stabilized by a kinase-inactive CDK2, using reticulocyte lysate-translated proteins *in vitro* and a promoter turn-off assay *in vivo*. Variation of the above assays like using bacterially expressed GST-CDK2 and GST-CDK2(K33R) in the *in vitro* degradation assay also produced the same conclusion.[2] We also showed that a kinase-inactive mutant of CDC2 and the CDK inhibitor $p21^{CIP1/WAF1}$ could also inhibit the degradation of cyclin A[2]. We think that either the lack of cyclin A phosphorylation by CDK2(K33R) inhibited cyclin A degradation or the expression of CDK2(K33R) blocked the cell cycle and hence inhibited the degradation of cyclin A. Given that cyclin E (which binds to the same CDK partner as cyclin A) is regulated by CDK2-dependent degradation (23), we studied the obvious possibility that autophosphorylation of cyclin A by CDK2/CDC2 may be required for its degradation.

We think that Ser-154 is the CDK2 phosphorylation site in cyclin A because of the following: (*a*) Ser-154 is the only Ser/Thr-Pro site in cyclin A; (*b*) mutation S154A abolished the phosphorylation of by CDK *in vitro*; (*c*) mutation S154A abolished most of the phosphorylation of cyclin A in the cell. Although Ser-154 is likely to be the only CDK phosphorylation site in cyclin A, Ser-154 is clearly not the only phosphorylation site in cyclin A in the cell (Fig. 5). Although Ser-154 phosphorylation is not involved in the degradation of cyclin A, it is possible that the phosphorylation of other sites in cyclin A by other protein kinases may target cyclin A for degradation or affect the activity of cyclin A.

What other explanations are there if direct phosphorylation of cyclin A by CDK2 does not explain the stabilization of cyclin A when expressed with CDK2(K33R)? One line of possibility is that CDK2(K33R) affected the phosphorylation of cyclin A-CDK toward other substrates (other than cyclin A itself). It is possible that phosphorylation of other substrates by cyclin

A-CDK is required for the degradation of cyclin A.

An alternative interpretation of the CDK2(K33R) experiments is that other cyclins were inhibited by CDK2(K33R), which in turn affected the degradation of cyclin A. CDK2(K33R) can bind to both cyclin A and cyclin E and act as dominant-negative mutants for both cyclins. It is conceivable that cyclin E-CDK2 activity may be required for cyclin A degradation. However, since CDK2(K33R) inhibited both the activity of cyclin E as well as cyclin E degradation (presumably through inhibition of Thr-380 phosphorylation), the CDK2(K33R) results are also consistent with the possibility that cyclin E degradation (inactivation) is required for cyclin A degradation. We think that these explanations involving cyclin E are unlikely, because the kinase-inactive CDC2 mutant can inhibit cyclin A but not cyclin E but nevertheless still abolished the degradation of cyclin A (Fig. 2*C*). Interpretations with the CDC2 kinase-inactive mutant is slightly complicated by the fact that although cyclin E was not affected, the activities of both cyclin A and cyclin B were inhibited. Similar speculation of whether the activity of cyclin B is involved in the degradation of cyclin A is equally complicated. Collectively, since direct phosphorylation of cyclin A by CDK2 is probably not involved in the destruction of cyclin A, it is likely that the stabilization of cyclin A by CDK2(K33R) was mainly due to a cell cycle effect. The experiments describe here that examined the stability of cyclin A and cyclin A(S154A) are still valid since they are conducted in the absence of CDK2(K33R) (Figs. 7 and 8).

For cyclin B1, the nuclear exporting sequence is responsible for its cytoplasmic localization during G_2. The nuclear export of cyclin B1 is mediated by binding of nuclear exporting sequence to the export mediator CRM1 (52–54). Phosphorylation of serine residues by CDK in the nuclear exporting sequence of cyclin B1 is important for its nuclear translocation, possibly through the disruption of CRM1-cyclin B1 interaction (53). Hence, this mechanism allows the localization of cyclin B1-CDC2 to the nucleus when the complexes are active. It is possible that phosphorylation of Ser-154 by CDKs may also affect the subcellular localization of cyclin A. However, we observed no gross difference in the subcellular localization between ectopic cyclin A and cyclin A(S154A).[2]

It is very interesting that the CDK phosphorylation site in cyclin A, Ser-154, is within the binding region of the recently identified protein XDRP1 (55). XDRP1 is related to the yeast protein Dsk2p and contains a ubiquitin-like domain at the N terminus. Binding of XDRP1 to cyclin A inhibits the degradation of cyclin A in *Xenopus* egg extracts. It would be interesting to see whether phosphorylation of Ser-154 affects the binding to XDRP1-like proteins to cyclin A. But since we found that S154A does not affect the stability of cyclin A, we do not expect that phosphorylation of Ser-154 has a significant effect on XDRP1 binding.

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